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# Biochemical and enzymatic characterization of decapsulated cysts and nauplii of the brine shrimp *Artemia* at different developmental stages

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**Abstract**

Decapsulated *Artemia* cysts were used to study the factors which induce the superior performance of live organisms as food for fish larvae. The biochemical composition, the in vitro protein digestibility, and the total proteolytic and trypsin activities in cysts and nauplii of *Artemia* were determined at different developmental stages as a function of incubation time. Six different incubation times were studied: 1, 6, 11, 16, 21 and 25 h of development, which cover cyst and early nauplii stages. The individual dry weight of *Artemia* decreased through development. The individual protein and lipid content ($\mu$g ind$^{-1}$) remained constant during development until the time of hatching after which they decreased slightly. However, no significant incubation effect was found. Small changes in amino acid and fatty acid composition were found during development, but is assumed that they are too small to be of nutritional importance to fish larvae. No major changes were observed in protease activities measured at acid and alkaline pH during the first 25 h of development. From the alkaline proteases, no significant change in trypsin activity was detected during cyst and early nauplii development. From the point of view of exogenous enzyme contribution to fish larvae, there seems to be no difference whether feeding decapsulated cysts or newly hatched nauplii, since no difference in qualitative protease composition was found

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during the first 25 h of *Artemia* development. The relative contribution of *Artemia* proteases to the digestion of food by fish larvae is discussed. © 1998 Elsevier Science B.V.

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## 1. Introduction

Larvae of nearly all marine and of many freshwater fish species require live planktonic organisms as first food. In fish culture, the use of live food organisms complicates the rearing techniques and increases the operational costs. For many fish species live food still gives better results in terms of growth and survival than artificial diets (Dabrowski, 1984). Several hypotheses have been proposed to explain this: e.g., food composition; structure and digestibility of dietary protein (Verreth, 1994); exogenous enzymes (Dabrowski and Glogowski, 1977; Lauff and Hofer, 1984); chemical and visual stimuli from the live food organisms (Kolkovski et al., 1995). Studies to understand those factors are complicated by the fact that any comparison between live and artificial diets are confounded by behavioral aspects. Decapsulated *Artemia* cysts have been proposed to solve the latter (Verreth et al., 1987).

Decapsulated *Artemia* cysts have been tested successfully for larval rearing of African catfish (Verreth et al., 1987; Pector et al., 1994) and common carp (Vanhaecke et al., 1990). The cysts of *Artemia* present some advantages to artificial diets because they combine the physical properties of a dry artificial feed and the nutritional value of live *Artemia* nauplii. The question is whether this hypothesis is true. The nutritional value of decapsulated cysts might be sensitive to the quality of the decapsulation and subsequent drying procedures. During the decapsulation process the cysts are hydrated, as a consequence, the reversibly interrupted metabolism of the *Artemia* embryo resumes. This might result in a food with a biochemical and nutritional composition that diverge from both dry cysts and live nauplii. From previous studies on the biochemical composition and nutritional value of *Artemia*, only Hines et al. (1980) followed the biochemical composition during the development of both cysts and nauplii. For future studies on the role of dietary enzymes in larval digestion, the characterization and quantitative estimation of digestive enzymes from decapsulated cysts of *Artemia* are necessary to determine the enzyme input from live food to fish larvae. However, a combined analysis of the biochemical and enzymatic changes in *Artemia* during cyst development is not available. The present study was designed to provide such a characterization for the same batch of cysts during their development.

## 2. Materials and methods

### 2.1. Hatching quality and cysts incubation

The hatching rate and hatching percentage of the *Artemia* cysts were determined following the procedure described by Sorgeloos et al. (1986). The same batch of *Artemia* cysts (INVE Aquaculture; type EG batch number 5335) from Great Salt Lake

(GSL), USA was used in all the analyses. The cysts were decapsulated with a solution of NaOCl, NaOH and water according to the method of Sorgeloos et al. (1986). In all analyses dry non-decapsulated cysts were used in parallel with decapsulated cysts to compare the changes in the embryo nutritional characteristics from the inactive dormant embryo to a cyst where the embryonic metabolism has been reactivated by incubation in seawater. After decapsulation, the cysts were incubated in hatching tanks with a conical bottom to start the embryonic development process. The incubation conditions were: temperature 28°C, salinity 33‰, approximately 1200 lux of continuous illumination at the water surface, and medium aeration from the bottom of the tank to maintain the cysts in suspension. Six different incubation times were used: 0, 5, 10, 15, 20, and 24 h, which encompass cyst and early nauplii development. After incubation samples for enzyme analysis were shock-frozen with liquid nitrogen and stored at −80°C. Samples for the biochemical and digestibility analyses were stored at −20°C and later freeze-dried before further processing.

### 2.2. Biochemical composition

Protein content was determined by Kjeldahl analysis according to ISO 5983 (ISO, 1979) procedures and calculated as nitrogen content multiplied by 6.25. Individual amino acids were measured with a Biotronic LC 5001 amino acid analyzer with a single column using an ion exchange resin. Amino acids were determined with ninhydrin as coloring reagent and quantified using an amino acid standard solution (Sigma AA-S-18), L-nor-leucine (Merck 24560) and L-tryptophan (Merck 8374). Cystine and methionine were analyzed after oxidation with performic acid and acid hydrolysis. Tryptophan was determined after alkaline hydrolysis. All other amino acids were determined after acid hydrolysis. The lipids in dry cysts samples were extracted following a modified procedure of Folch et al. (1957) using chloroform–methanol (2:1) and esterified with a mixture of 1% $H_2SO_4$ in methanol. After fat extraction and esterification a fatty acid methyl esther (FAME) analysis was done according to the method described by Coutteau and Sorgeloos (1995) to obtain the HUFA profile of cysts samples. Lipid classes were separated by high-performance thin-layer chromatography (HPTLC) using a single-dimension double-development method (Tocher and Harvie, 1988). The lipid classes were quantified by charring followed by densitometry using a Sharp JX-325 scanner supported with ImageMaster™ Software (modified from Olsen and Henderson, 1989). Total carbohydrate was determined by the phenol-sulfuric acid reaction (Dubois et al., 1956) using glucose as a standard. Ash content was determined by burning oven-dried samples in a muffle furnace at 550°C according to ISO 5984 (ISO, 1978). Individual dry weight was estimated by freeze-drying a known amount of cysts incubated in 2 l aerated containers at the different experimental times. The dry weight of the sample per volume was divided by the number of individuals counted in 250 $\mu$l samples taken during the incubation.

### 2.3. In vitro protein digestibility

A modified filtration method from Babinszky et al. (1990) was used to predict the protein digestibility in vitro of the cysts and nauplii samples. The samples were

incubated in a two-step procedure with enzymes. In the first step, the protein was digested with a solution of pepsin (Merck, 7190) and HCl (pepsin 2 g $l^{-1}$ in HCl 0.1 M, pH 1) for 1 h at 30°C. In the second step, the samples were further digested for 1 h with a solution of pancreatin (Merck, 7133), amylase (NOVO, Termamyl 120L), lipase (Sigma L3001) and bile salts (Sigma B8756) dissolved in a mixture of $Na_2HPO_4$ and $KH_2PO_4$. After digestion in the multi-enzyme system, the digested fraction was vacuum-filtrated and the amount of undigested crude protein was determined by the Kjeldhal method. From the nitrogen content of the original cysts sample and the undigested protein from this analysis, the in vitro digestible protein content was calculated.

### 2.4. Enzyme analyses

The total protease was measured in cysts and nauplii homogenates. For homogenate preparation 75 mg of sample was ground in a potter blender and dissolved in 750 $\mu$l of distilled water. After homogenization the samples were sonified for 30 s and centrifuged at 12 000 × $g$ during 10 min at 4°C. During preparation the homogenates were continuously kept on ice. Total protease activity was measured by a modified casein method from Walter (1984) using casein (10 mg $ml^{-1}$) dissolved in 0.1 M Tris buffer at pH 8. The protease activity was determined at different pH values of 3, 4, 6, 8 and 10. A citrate–phosphate buffer (0.1 M citric acid plus 0.2 M $Na_2HPO_4$) and a buffer composed of 0.2 M Tris and 0.1 N HCl were used to detect acidic and alkaline protease, respectively, with L-tyrosine as a standard. The assay mixture, which consisted of 400 $\mu$l casein solution, 400 $\mu$l buffer and 200 $\mu$l supernatant of homogenate, was incubated at 30°C for 60 min, the reaction was stopped with trichloroacetic acid (0.3 M). After cooling on ice for 15 min, the assay mixtures were centrifuged at 1700 × $g$ for 10 min at 4°C and the absorbance of the supernatant was measured at 280 nm with a spectrophotometer. The results are expressed as mg tyrosine liberated per g protein in sample per 60 min. Protein was determined by Kjeldahl analysis.

The trypsin activity was determined using the modified method of Hofer and Köck (1989) and Bergmeyer (1974) using the substrate BAPNA (*Nα*-benzoyl-DL-arginine-4-nitroanilide, Merck 1670). The homogenate preparation for the trypsin measurements was similar to the casein assay with the difference that 100 mg of cyst or nauplii sample was ground and dissolved in 500 $\mu$l of buffer. The assay mixture consisted of 3.9 mM BAPNA in a 0.1 M Tris buffer pH 8 with 0.2 M $CaCl_2$. The enzymatic reaction was started by adding BAPNA to a mixture of buffer and homogenate. The change in absorbance during 5 min was measured at 405 nm. Trypsin activity is reported as Units $g^{-1}$ of sample (1 U = 1 $\mu$mol BAPNA converted per min).

### 2.5. Data analysis

Four samples per incubation time were analyzed for total proteolytic activity and at least three for trypsin activity. Four samples per incubation time were analyzed for individual amino acid content except for cystine, methionine, tyrosine, histidine, phenylalanine and tryptophan which were determined with two samples. For protein, lipid, carbohydrate, fatty acid and ash content, protein digestibility and individual dry weight

three samples per incubation time were analyzed. Lipid classes were determined with two samples per treatment. The change in individual weight was also considered when comparing changes in any nutritional component during the development of *Artemia* from cyst to nauplii. The data expressed as relative values were tested for normal distribution and submitted to square root arcsine transformation before a one-way ANOVA was performed. When significant differences were found, the means were compared with Duncan's multiple range test ($P < 0.05$). The data expressed as absolute values were submitted to a non-parametric one-way ANOVA (Kruskal–Wallis test). The statistical analyses were done with SAS statistical software package (SAS Institute).

Prior to decapsulation, the cysts were hydrated for 1 h and this hydration reactivates the metabolism of the dormant embryo in the cysts, therefore in the results, 1 h is added to all incubation times and is indicated as hours of development.

## 3. Results

### 3.1. Hatching quality

The hatching efficiency corresponded to 144 377 nauplii $g^{-1}$ cysts and the hatching percentage was 70.1 ± 5.1%. From the hatching curve, $T_{10}$ (time of 10% hatching) and

Table 1
Proximate composition, individual dry weight and in vitro protein digestibility of *Artemia* at different developmental stages as function of incubation time

| | Undecapsulated cysts | Time of development (h) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 6 | 11 | 16 | 21 | 25 |
| *Protein* | | | | | | | |
| % dw | $55.8 \pm 0.1$ | $50.6 \pm 0.1^{e}$ | $53.8 \pm 0.1^{d}$ | $54.7 \pm 0.1^{c}$ | $55.7 \pm 0.2^{b}$ | $56.3 \pm 0.1^{a}$ | $56.2 \pm 0.0^{a}$ |
| $\mu g\ ind^{-1}$ * | $2.70 \pm 0.1$ | $1.73 \pm 0.07$ | $1.77 \pm 0.08$ | $1.67 \pm 0.07$ | $1.70 \pm 0.08$ | $1.48 \pm 0.06$ | $1.30 \pm 0.06$ |
| *Lipid* | | | | | | | |
| % dw | $11.2 \pm 0.2$ | $14.7 \pm 0.1^{d}$ | $16.2 \pm 0.5^{c}$ | $16.4 \pm 0.3^{bc}$ | $16.9 \pm 0.3^{b}$ | $18.0 \pm 0.2^{a}$ | $17.0 \pm 0.3^{b}$ |
| $\mu g\ ind^{-1}$ * | $0.54 \pm 0.03$ | $0.51 \pm 0.02$ | $0.53 \pm 0.03$ | $0.51 \pm 0.02$ | $0.52 \pm 0.02$ | $0.48 \pm 0.02$ | $0.40 \pm 0.02$ |
| *Carbohydrate* | | | | | | | |
| % dw | $6.9 \pm 0.7$ | $6.6 \pm 0.5^{a}$ | $6.6 \pm 1.1^{a}$ | $4.2 \pm 0.3^{b}$ | $4.3 \pm 0.2^{b}$ | $4.3 \pm 0.7^{b}$ | $3.6 \pm 0.4^{b}$ |
| $\mu g\ ind^{-1}$ * | $0.33 \pm 0.01$ | $0.23 \pm 0.01$ | $0.22 \pm 0.01$ | $0.13 \pm 0.01$ | $0.14 \pm 0.01$ | $0.12 \pm 0.01$ | $0.09 \pm 0.01$ |
| *Ash* | | | | | | | |
| % dw | $5.9 \pm 0.2$ | $10.6 \pm 0.3^{a}$ | $11.1 \pm 0.2^{a}$ | $9.0 \pm 0.2^{bc}$ | $9.8 \pm 0.8^{ab}$ | $7.2 \pm 0.1^{d}$ | $7.6 \pm 1.2^{cd}$ |
| $\mu g\ ind^{-1}$ * | $0.29 \pm 0.01$ | $0.36 \pm 0.01$ | $0.36 \pm 0.01$ | $0.28 \pm 0.01$ | $0.30 \pm 0.01$ | $0.19 \pm 0.01$ | $0.18 \pm 0.01$ |
| *Individual dry weight* | | | | | | | |
| $\mu g$ * | $4.83 \pm 0.2$ | $3.42 \pm 0.2$ | $3.28 \pm 0.2$ | $3.06 \pm 0.1$ | $3.05 \pm 0.1$ | $2.62 \pm 0.1$ | $2.31 \pm 0.1$ |
| *Digestible protein* | | | | | | | |
| % Total protein | – | $82.8 \pm 1.3$ | $79.7 \pm 7.1$ | $81.5 \pm 4.0$ | $79.5 \pm 2.8$ | $82.5 \pm 1.0$ | $77.8 \pm 1.6$ |

The time of development is the time of incubation plus 1 h of hydration time during the decapsulation process. Means in the same row with the same superscript are not significantly different ($P > 0.05$) (% values).
* No significant incubation effect was detected ($P > 0.05$).

$T_{50}$ (50% hatching) were graphically derived and corresponded to 14.5 and 17.3 h, respectively. The cysts showed 90% of their maximum hatchability ($T_{90}$) at 21.8 h. The hatching synchrony ($T_s = T_{90} - T_{10}$) was 7.3 h.

The composition of the samples taken from each of the incubation times were: 100% of cysts for 1, 6 and 11 h of development; 35% cysts and 65% nauplii (umbrella stages included) for 16 h; 33% cysts and 67% nauplii for 21 h; 30% cysts and 70% nauplii for 25 h. The latter 30% cysts can be regarded as unhatchable cysts which were present in all samples.

### 3.2. Proximate analysis

The protein, lipid, carbohydrate and ash composition of cysts and nauplii of *Artemia* are given in Table 1 in relative (% dw) and absolute values ($\mu$g ind$^{-1}$). The relative concentration in the dry matter (% dw) of all four nutritional components changed significantly ($P < 0.05$) during cyst development. However, for the absolute values no significant incubation effect was found ($P > 0.05$). In undecapsulated cysts the individual protein content was higher than in all subsequent developing stages. However,

Table 2
Amino acid composition (g 100 g$^{-1}$ protein) of *Artemia* at different developmental stages as function of incubation time

| Amino acid | Undecapsulated cysts | Time of development (h) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 6 | 11 | 16 | 21 | 25 |
| Aspartic acid | 8.6±0.2 | 7.7±0.2 | 7.5±0.4 | 7.4±0.0 | 7.5±0.1 | 7.6±0.3 | 7.6±0.1 |
| Threonine | 4.1±0.0 | 4.3±0.2 | 4.4±0.1 | 4.2±0.1 | 4.2±0.1 | 4.3±0.1 | 4.3±0.1 |
| Serine | 6.3±0.1 | 5.7±0.1$^{a}$ | 5.9±0.1$^{a}$ | 5.5±0.0$^{b}$ | 5.5±0.0$^{b}$ | 5.5±0.1$^{b}$ | 5.5±0.0$^{b}$ |
| Glutamic acid | 10.3±0.4 | 11.0±0.4$^{ab}$ | 11.1±0.2$^{ab}$ | 10.3±0.1$^{c}$ | 10.9±0.1$^{b}$ | 11.0±0.1$^{ab}$ | 11.2±0.1$^{a}$ |
| Proline | 5.0±0.2 | 3.6±0.1$^{c}$ | 3.6±0.1$^{c}$ | 3.8±0.2$^{b}$ | 4.0±0.2$^{ab}$ | 4.0±0.1$^{a}$ | 4.2±0.1$^{a}$ |
| Glycine | 4.0±0.1 | 3.9±0.0$^{ab}$ | 3.9±0.1$^{b}$ | 3.9±0.0$^{b}$ | 4.0±0.0$^{a}$ | 4.0±0.1$^{a}$ | 4.0±0.0$^{a}$ |
| Alanine | 5.1±0.1 | 4.1±0.0$^{c}$ | 4.3±0.1$^{b}$ | 4.2±0.0$^{c}$ | 4.2±0.0$^{c}$ | 4.3±0.1$^{b}$ | 4.4±0.0$^{a}$ |
| Cystine | 1.3±0.0 | 1.1±0.1 | 1.0±0.0 | 1.0±0.0 | 1.0±0.0 | 1.1±0.0 | 1.1±0.0 |
| Valine | 4.9±0.1 | 4.9±0.1 | 4.8±0.1 | 4.8±0.1 | 4.8±0.1 | 4.8±0.1 | 4.9±0.1 |
| Methionine | 2.1±0.0 | 2.5±0.1 | 2.2±0.4 | 2.4±0.0 | 2.4±0.0 | 2.4±0.0 | 2.3±0.0 |
| Isoleucine | 4.4±0.1 | 4.8±0.0$^{a}$ | 4.7±0.1$^{ab}$ | 4.6±0.0$^{b}$ | 4.6±0.1$^{b}$ | 4.6±0.1$^{b}$ | 4.7±0.0$^{ab}$ |
| Leucine | 6.0±0.2 | 6.6±0.1$^{a}$ | 6.5±0.1$^{ab}$ | 6.3±0.0$^{c}$ | 6.4±0.1$^{bc}$ | 6.4±0.1$^{bc}$ | 6.5±0.1$^{abc}$ |
| Tyrosine | 5.2±0.1 | 3.3±0.0$^{ab}$ | 3.1±0.2$^{b}$ | 3.3±0.0$^{ab}$ | 3.3±0.0$^{ab}$ | 3.3±0.0$^{ab}$ | 3.4±0.0$^{a}$ |
| Phenylalanine | 3.7±0.0 | 3.9±0.0$^{a}$ | 3.8±0.0$^{bc}$ | 3.8±0.0$^{c}$ | 3.8±0.0$^{c}$ | 3.8±0.0$^{bc}$ | 3.9±0.0$^{ab}$ |
| Histidine | 2.7±0.1 | 2.7±0.0$^{a}$ | 2.7±0.0$^{a}$ | 2.6±0.0$^{b}$ | 2.4±0.0$^{c}$ | 2.5±0.0$^{c}$ | 2.5±0.0$^{bc}$ |
| Lysine | 7.1±0.2 | 7.4±0.1$^{a}$ | 7.3±0.1$^{ab}$ | 6.9±0.1$^{d}$ | 7.2±0.0$^{c}$ | 7.2±0.1$^{bc}$ | 7.3±0.1$^{ab}$ |
| Arginine | 6.4±0.2 | 7.0±0.0$^{a}$ | 7.0±0.1$^{b}$ | 6.7±0.0$^{d}$ | 6.7±0.0$^{cd}$ | 6.7±0.1$^{cd}$ | 6.8±0.0$^{c}$ |
| Tryptophan | 0.9±0.1 | 1.3±0.1$^{a}$ | 1.2±0.0$^{ab}$ | 1.2±0.0$^{ab}$ | 1.1±0.1$^{b}$ | 1.2±0.1$^{ab}$ | 1.2±0.1$^{ab}$ |
| Total amino acids | 88.1±1.5 | 85.8±0.6$^{a}$ | 85.0±0.5$^{ab}$ | 82.9±0.6$^{d}$ | 84.0±0.3$^{bc}$ | 84.7±1.0$^{cd}$ | 85.8±0.5$^{a}$ |

The time indicated as hours of development is the time of incubation plus 1 h of hydration time during the decapsulation process.
Means in the same row with the same superscript are not significantly different ($P > 0.05$).
Standard deviation values were rounded to decimals.

during incubation (e.g., embryonal development) individual protein and lipid contents remained constant, exceptions were at 21 and 25 h which corresponded with the times of the highest presence of nauplii in the samples. Individual carbohydrate and ash contents also decreased towards the naupliar stage, attaining low values at 25 h of development. The individual dry weight was highest for undecapsulated cysts, and decreased during development (Table 1).

Table 3
Fatty acid composition (mg $g^{-1}$ dw) of *Artemia* at different developmental stages as function of incubation time

| Fatty acid | Undecapsulated cysts | Time of development (h) 1 | 6 | 11 | 16 | 21 | 25 |
|---|---|---|---|---|---|---|---|
| 14:0 | 1.3±0.1 | 1.6±0.1$^{a}$ | 1.8±0.1$^{a}$ | 1.7±0.1$^{a}$ | 1.8±0.1$^{a}$ | 1.7±0.4$^{a}$ | 1.2±0.1$^{b}$ |
| 14:1$n-5$ | 1.1±0.1 | 1.3±0.1$^{ab}$ | 1.5±0.1$^{a}$ | 1.5±0.1$^{a}$ | 1.6±0.1$^{a}$ | 1.4±0.4$^{a}$ | 1.1±0.0$^{b}$ |
| 15:0 | 0.3±0.0 | 0.4±0.0$^{a}$ | 0.4±0.0$^{a}$ | 0.4±0.0$^{a}$ | 0.4±0.0$^{a}$ | 0.4±0.1$^{b}$ | 0.3±0.0$^{c}$ |
| 15:1$n-5$ | 0.6±0.1 | 0.7±0.0$^{ab}$ | 0.8±0.0$^{a}$ | 0.8±0.1$^{a}$ | 0.8±0.1$^{a}$ | 0.8±0.2$^{a}$ | 0.6±0.0$^{b}$ |
| 16:0 | 12.7±1.3 | 16.1±0.4$^{a}$ | 17.8±0.8$^{a}$ | 17.7±0.8$^{a}$ | 18.6±1.4$^{a}$ | 16.5±4.0$^{a}$ | 13.2±0.6$^{b}$ |
| 16:1$n-7$ | 3.9±0.4 | 5.0±0.1$^{a}$ | 5.6±0.2$^{a}$ | 5.6±0.2$^{a}$ | 5.9±0.5$^{a}$ | 5.2±1.3$^{a}$ | 4.1±0.2$^{b}$ |
| 17:0 | 0.9±0.1 | 1.1±0.1$^{ab}$ | 1.2±0.1$^{a}$ | 1.1±0.1$^{a}$ | 1.3±0.1$^{a}$ | 1.1±0.3$^{ab}$ | 0.9±0.0$^{b}$ |
| 17:1$n-7$ | 1.1±0.1 | 1.4±0.0$^{ab}$ | 1.6±0.1$^{a}$ | 1.6±0.1$^{a}$ | 1.7±0.1$^{a}$ | 1.5±0.4$^{a}$ | 1.2±0.1$^{b}$ |
| 18:0 | 4.4±0.4 | 5.3±0.1$^{ab}$ | 5.9±0.3$^{a}$ | 5.8±0.2$^{a}$ | 6.3±0.5$^{a}$ | 5.7±1.3$^{ab}$ | 4.8±0.2$^{b}$ |
| 18:1$n-9$ | 19.1±1.9 | 24.2±0.5$^{ab}$ | 26.9±1.2$^{a}$ | 26.7±1.2$^{a}$ | 28.3±2.0$^{a}$ | 25.1±6.2$^{a}$ | 20.3±0.8$^{b}$ |
| 18:1$n-7$ | 6.7±0.7 | 8.4±0.2$^{bc}$ | 9.4±0.3$^{ab}$ | 9.6±0.4$^{ab}$ | 10.3±0.7$^{a}$ | 9.2±2.3$^{ab}$ | 7.4±0.3$^{c}$ |
| 18:2 $n-6-t$ | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.2±0.0 | 0.2±0.1 | 0.3±0.1 | 0.2±0.1 |
| 18:2 $n-6-c$ | 5.5±0.5 | 6.9±0.2$^{a}$ | 7.6±0.3$^{a}$ | 7.6±0.3$^{a}$ | 8.1±0.6$^{a}$ | 7.1±1.8$^{a}$ | 5.7±0.3$^{b}$ |
| 18:3$n-6$ | 0.5±0.1 | 0.6±0.0$^{ab}$ | 0.7±0.1$^{a}$ | 0.6±0.1$^{ab}$ | 0.6±0.1$^{a}$ | 0.6±0.1$^{ab}$ | 0.5±0.1$^{b}$ |
| 18:3$n-3$ | 27.3±2.8 | 34.2±0.8$^{ab}$ | 37.9±1.1$^{a}$ | 37.8±1.7$^{a}$ | 39.6±2.5$^{a}$ | 35.6±9.0$^{a}$ | 28.6±1.2$^{b}$ |
| 18:4$n-3$ | 4.6±0.5 | 5.6±0.1$^{a}$ | 6.2±0.2$^{a}$ | 6.0±0.3$^{a}$ | 6.2±0.4$^{a}$ | 5.5±1.4$^{a}$ | 4.5±0.2$^{b}$ |
| 20:0 | 0.1±0.0 | 0.1±0.1 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 |
| 20:1$n-9$ | 0.4±0.1 | 0.4±0.0$^{b}$ | 0.5±0.1$^{ab}$ | 0.5±0.1$^{a}$ | 0.5±0.1$^{a}$ | 0.5±0.1$^{ab}$ | 0.4±0.0$^{b}$ |
| 20:4$n-6$ | 0.6±0.0 | 0.7±0.1$^{ab}$ | 0.8±0.1$^{a}$ | 0.8±0.1$^{a}$ | 0.8±0.1$^{a}$ | 0.8±0.2$^{ab}$ | 0.6±0.1$^{b}$ |
| 20:3$n-3$ | 0.6±0.0 | 0.7±0.0$^{ab}$ | 0.8±0.1$^{ab}$ | 0.8±0.0$^{a}$ | 0.8±0.1$^{a}$ | 0.8±0.2$^{ab}$ | 0.6±0.1$^{b}$ |
| 20:4$n-3$ | 0.7±0.1 | 0.8±0.0$^{ab}$ | 0.9±0.0$^{a}$ | 0.8±0.1$^{ab}$ | 0.9±0.1$^{a}$ | 0.8±0.2$^{b}$ | 0.7±0.0$^{b}$ |
| 22:0 | 0.1±0.0 | 0.2±0.1 | 0.2±0.1 | 0.2±0.0 | 0.2±0.1 | 0.3±0.1 | 0.2±0.0 |
| 20:5$n-3$ | 3.2±0.3 | 4.2±0.2$^{a}$ | 4.7±0.2$^{a}$ | 4.7±0.2$^{a}$ | 4.9±0.3$^{a}$ | 4.4±1.1$^{a}$ | 3.5±0.2$^{b}$ |
| 22:6$n-3$ | 0.1±0.0 | 0.1±0.0 | 0.1±0.1 | tr | tr | tr | tr |
| $\Sigma n-3>$ or = 20:3$n-3$ | 4.6 | 5.8 | 6.5 | 6.3 | 6.6 | 6.0 | 4.8 |
| $\Sigma n-6>$ or = 18:2$n-6-t$ | 6.8 | 8.4 | 9.3 | 9.2 | 9.7 | 8.8 | 7.0 |
| Total FAME | 100.0±7.1 | 125.9±2.9$^{ab}$ | 139.4±5.0$^{a}$ | 139.3±6.0$^{a}$ | 146.8±10.1$^{a}$ | 130.8±32.2$^{a}$ | 105.6±4.3$^{b}$ |

The time of development is the time of incubation plus 1 h of hydration time during the decapsulation process.
Means in the same row with the same superscript are not significantly different ($P > 0.05$).
Standard deviation values were rounded to decimals.
tr = Trace.

Table 4
Lipid classes (mg $g^{-1}$ dw) in *Artemia* at different developmental stages as function of incubation time

| Lipid class | Undecapsulated cysts | Time of development (h) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 6 | 11 | 16 | 21 | 25 |
| *Polar* | | | | | | | |
| Sphingomyelin | 0.5±0.0 | nd | 2.7±0.0$^{a}$ | 3.0±1.2$^{a}$ | 2.9±0.1$^{a}$ | 3.0±0.3$^{a}$ | 2.6±0.1$^{a}$ |
| Lysophosphatidylcholine | nd | nd | nd | 0.8±0.1$^{a}$ | 0.4±0.1$^{b}$ | 0.8±0.1$^{a}$ | 0.7±0.0$^{b}$ |
| Phosphatidylcholine | 16.7±1.2 | 24.7±1.8$^{a}$ | 24.3±1.2$^{a}$ | 23.4±0.2$^{ab}$ | 23.5±0.5$^{ab}$ | 24.4±0.1$^{a}$ | 21.5±0.3$^{b}$ |
| Phosphatidylserine | 0.8±0.2 | 0.5±0.2$^{c}$ | 2.9±0.0$^{b}$ | 2.8±0.0$^{b}$ | 2.8±0.4$^{b}$ | 4.8±0.3$^{a}$ | 3.0±0.1$^{b}$ |
| Phosphatidylinositol | 2.6±0.4 | 3.9±0.4$^{c}$ | 4.5±2.0$^{bc}$ | 6.0±0.0$^{ab}$ | 6.0±0.1$^{ab}$ | 7.8±0.0$^{a}$ | 6.6±0.2$^{ab}$ |
| Phosphatidic acid + cardiolipine | 2.5±0.3 | 2.8±0.2$^{c}$ | 3.9±1.6$^{bc}$ | 4.9±0.2$^{ab}$ | 5.1±0.1$^{ab}$ | 6.2±0.4$^{a}$ | 4.8±0.1$^{ab}$ |
| Phosphatidylethanolamine | 7.6±0.5 | 11.4±0.4$^{d}$ | 13.1±1.0$^{bc}$ | 13.7±0.2$^{bc}$ | 14.3±0.2$^{ab}$ | 15.1±0.4$^{a}$ | 12.4±0.3$^{cd}$ |
| *Neutral* | | | | | | | |
| Pigments | 5.0±0.2 | 4.6±0.1$^{b}$ | 7.6±3.8$^{ab}$ | 10.1±0.6$^{a}$ | 9.9±0.4$^{a}$ | 9.9±0.9$^{a}$ | 11.5±0.3$^{a}$ |
| Cholesterol | 8.4±0.2 | 11.5±0.5$^{b}$ | 13.0±0.6$^{a}$ | 11.2±0.3$^{b}$ | 12.0±0.9$^{ab}$ | 12.1±0.2$^{ab}$ | 11.0±0.6$^{b}$ |
| Free fatty acids | 4.6±0.1 | 3.5±0.3$^{d}$ | 6.1±4.5$^{cd}$ | 8.9±0.3$^{bc}$ | 9.7±0.4$^{bc}$ | 13.1±1.2$^{b}$ | 23.1±1.4$^{a}$ |
| Triglycerides | 49.9±1.1 | 67.9±2.0$^{a}$ | 72.0±9.1$^{a}$ | 65.1±0.3$^{a}$ | 64.9±1.4$^{a}$ | 65.0±0.1$^{a}$ | 52.5±0.2$^{b}$ |
| Cholesterol esters | 14.3±0.9 | 16.3±1.4 | 14.1±7.8 | 8.9±0.4 | 10.8±0.5 | 11.9±1.2 | 12.9±0.0 |
| Total polar lipid | 29.7±2.3 | 43.3±3.0$^{c}$ | 49.2±8.0$^{bc}$ | 54.6±0.4$^{ab}$ | 55.1±0.1$^{ab}$ | 62.1±0.6$^{a}$ | 51.6±0.4$^{bc}$ |
| Total neutral lipid | 82.2±2.3 | 103.8±3.1$^{a}$ | 112.8±7.9$^{a}$ | 104.2±0.5$^{c}$ | 107.3±0.0$^{a}$ | 112.0±0.9$^{a}$ | 111.0±0.9$^{a}$ |

The time indicated as hours of development is the time of incubation plus 1 h of hydration time during the decapsulation process.
Means in the same row with the same superscript are not significantly different ($P > 0.05$).
nd = Not detected.

### 3.3. Amino acid composition

In general, small differences were found among individual amino acids (Table 2). The differences were larger for lysine and arginine for which smaller values were measured at 11 h of development, just before hatching. No significant differences were detected for aspartic acid, threonine, cystine, valine and methionine. Tryptophan was detected at all times of development and its values ranged from 0.9 g 100 $g^{-1}$ protein in undecapsulated cysts up to 1.3 g 100 $g^{-1}$ protein at 1 h. The total amino acid content remained constant during development with exceptions at 11 and 16 h when it decreased and corresponded to the time before and after hatching, respectively.

### 3.4. Fatty acid composition and lipid classes

As indicated in Table 3, the fatty acid composition during cysts and nauplii development presented small differences. A slight decreasing trend was detected in samples at 25 h of incubation; for most of the fatty acids, the values of nauplii were

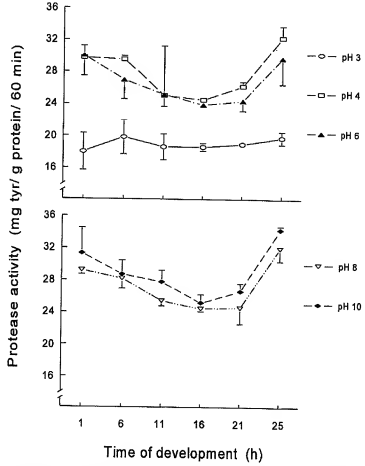


Fig. 1. Total proteolytic activity during early development of cysts and nauplii of *Artemia* measured at acid and alkaline pH. The time of development is the time of incubation plus 1 h of hydration during the decapsulation process.

smaller than in the previous developmental stages. Most of the lipid classes remained constant during development (Table 4). Free fatty acids increased strongly towards 25 h of development. Total polar lipid increased towards 21 h of development, while the total neutral lipid did not vary.

### 3.5. In vitro protein digestibility

High protein digestibility (expressed as percentage of total protein content) was found for all the developmental stages (Table 1). It ranged from 77.8% at 25 h to 82.8% at 1 h. No difference in digestibility was found between cysts and nauplii samples

### 3.6. Enzyme analyses

The total proteolytic activity in cysts and nauplii measured at acid and alkaline pH is presented in Fig. 1. The protease activity in undecapsulated cysts at pH 8 was 11.9 mg Tyr $g^{-1}$ protein 60 $min^{-1}$ compared to 28.7 for 1 h incubated cysts. This indicates a sharp increase in enzymatic activity after the reactivation of the embryo metabolism by hydration of cysts. The protease activity during cyst and early nauplii development differed significantly when measured at pH 4, 6, 8 and 10. The lowest protease activity was observed around 16 h of development. The effect of pH on the measurement of protease activity was similar in all the treatments, i.e., only the values at acid pH were lower. For trypsin activity (Fig. 2) no significant differences were found between the studied developmental stages.

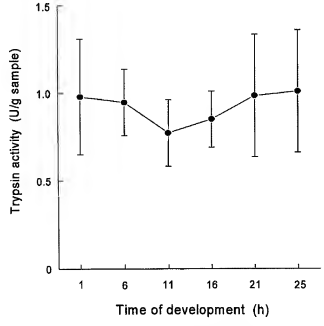


Fig. 2. Trypsin activity during early development of cysts and nauplii of *Artemia*. The time of development is the time of incubation plus 1 h of hydration during the decapsulation process.

## 4. Discussion

The hatching rate and hatching synchrony obtained in the present study indicate that good quality cysts were used.

The nutritional quality in *Artemia* varies considerably. This variation might be related to the geographical origin of *Artemia* (Léger et al., 1986), to differences among different batches of cysts from the same origin, and to the methods of analysis. Because of this variation, in nutritional studies with fish larvae it is important to determine the biochemical composition of the live food being used. The high protein content in undecapsulated cysts is due to the presence of the chorion or external layer composed of lipoprotein impregnated with chitin and hematin (Sorgeloos et al., 1986). The chorion is removed during decapsulation, resulting in a lower protein content and individual weight measured in decapsulated cysts and in newly hatched nauplii. The proximate composition in cysts and nauplii varied according to changes in individual dry weight. To determine the nutritional quality of *Artemia* it is important to study changes in proximate composition on individual weight basis and not exclusively in relative terms (% dw). The individual protein and fat content, and most notoriously the individual carbohydrate content decreased towards the naupliar stage. However no significant differences in individual proximate composition were found during development. All constituents were used during development, most probably for energy purposes. In general, cysts constitute a food item with a consistent biochemical composition. During development from cyst to nauplius, no drastic changes in the biochemical properties occur. Greater changes in biochemical composition might be expected between different strains of *Artemia* (Léger et al., 1986).

*Artemia* nauplii of different origin have different amino acid profiles (Watanabe et al., 1983; Dendrinos and Thorpe, 1987). For GSL cysts, Landau and Riehm (1985) found a higher relative amount of tryptophan (8.3% total amino acids) compared to 1.1% in this study. Since no other authors have analyzed or found tryptophan, no definitive conclusion can be made with regard to its proportional presence in *Artemia*. Methionine was not detected in starved adults of *Artemia* (Claus et al., 1979). However, in the present study methionine was detected in cysts and in early nauplii. According to Watanabe et al. (1983) *Artemia* nauplii are deficient in histidine, methionine, phenylalanine and threonine, whereas adults are rich in all essential amino acids. In the present study, that deficiency was not evident, at least in quantitative terms. The small decrease in individual fat content from cysts to early nauplii is probably due to the utilization of lipid reserves. This, together with the decrease in individual protein content, reflects the need of feeding the nauplii with microalgae or yeast when *Artemia* are being used as food for fish larvae. No previous reports exist on the HUFA composition of *Artemia* during development of cysts and only the 1 and 21 h samples, which corresponded to decapsulated cysts and early nauplii, respectively, can be compared with previous data on GSL *Artemia*. It is concluded that the small changes in amino acid and fatty acid composition during the development of *Artemia* cyst and early nauplii might have no relevance regarding its nutritional value as food for fish larvae. The total amino acid content of samples at 1 h (cysts) and 25 h (naupliar stage) was not significantly different, and the total fatty acid content of nauplii was lower than in cysts. However,

*Artemia* nauplii yielded higher growth than cysts when fed to catfish larvae (García-Ortega et al., 1995). The superior performance of live food is probably more related to food intake and digestibility than to the biochemical composition of the food.

No major changes occurred in protease activities during cyst development. There was a small tendency for a transient decrease around 16 h, coinciding with the hatching time. However, this might not represent a relevant aspect with respect to the nutritional importance for digestive processes in fish larvae. The protease composition did not differ between decapsulated cysts and nauplii of *Artemia*. As a consequence, as far as it concerns the hypothetical contribution of exogenous enzymes to fish larvae, there is no difference in feeding decapsulated cysts or early nauplii. From the protease activity in *Artemia* embryos and nauplii, over 90% is related to a cysteine protease that is used as hatching enzyme, in yolk utilization and as a digestive enzyme when the nauplii begins to eat (Warner et al., 1995). The importance and potential activity of this protease for fish larvae depends on the pH of the larval gut. Cysteine proteases are not active at alkaline pH (Warner and Shridhar, 1985). Thus the supply of these proteases might be meaningless to the fish larvae if the pH in the larval intestine is around 8.

Recently, Cahu et al. (1995) and Kurokawa et al. (1998) demonstrated that proteases derived from live food had only a small contribution to the enzymatic activity measured in sea bass and sardine larvae, respectively. A post-hatching activation of trypsin-like proteases in *Artemia* (Pan et al., 1991) might not be of much importance to the nutritional quality of nauplii, and thus can not be used as an argument to use *Artemia* nauplii instead of decapsulated cysts as food for fish larvae. More important differences might be expected from other factors such as the structure and digestibility of proteins and the food intake stimulation derived from live nauplii. Additional information in this regard is required to define the factors that influence the better utilization of live food by the fish larvae when compared to artificial diets.

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